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FORMULATION AND DELIVERY OF CPG-ODN IN CHICKENS AS AN IMMUNOSTIMULANT AND ADJUVANT

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"Formulation and delivery of CpG-ODN in chickens as an immunostimulant and adjuvant."

By

Susantha Gomis

Department of Veterinary Pathology
Western College of Veterinary Medicine
University of Saskatchewan
52, Campus Drive
Saskatoon. Saskatchewan
S7N 5B4

PH: (306) 966-7299
FX: (306) 966-7439

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(a) SUMMARY

Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to be effective immunoprotective agents and vaccine adjuvants in a variety of bacterial, viral and protozoan diseases in different animal species. The objective of this study was to compare the immune response of chickens to a killed *E. coli* vaccine combined with oil in water emulsion or with CpG-ODN. Birds were vaccinated with killed *E. coli* antigens with either 10 or 50 μg of CpG-ODN on days 10 and 20 of age. At day 30, a virulent isolate of homologus *E. coli* was applied on a scratch site on the caudal abdominal region. Birds were examined for 10 days post *E. coli* challenge, and pathological and bacteriological assessments were conducted on all birds that were either found dead or euthanized. The *E. coli* vaccine group that received no CpG-ODN had a survival rate of 65%. In contrast, groups that received the vaccine with CpG-ODN adjuvant had significantly higher survival rate of 92% ($p<0.01$) with isolation of low numbers of *E. coli* from internal organs. Total IgG against *E. coli* antigens was highest in groups that received CpG-ODN as an adjuvant. Birds that received vaccine containing CpG-ODN had minimal inflammatory reaction without tissue necrosis at the injection site. Severe tissue necrosis was present in birds that received vaccine containing oil in water emulsion adjuvant. This study demonstrated that CpG-ODN is an effective vaccine adjuvant in chickens and results in minimal tissue destruction. This study is the first time that CpG-ODN has been demonstrated to produce an adaptive immune response, at a significant level, against an extracellular bacterial infection in chickens.

(b) INTRODUCTION

The identification of events and signals that can boost immune responses to pathogens has been an active area of investigation for many decades. These studies have led to the discovery of various immunomodulators, as well as to the identification of cytokine pathways that allow cellular communication to direct the immune response. Initial studies demonstrated that specific DNA sequences from *Mycobacterium bovis* were effective in stimulating the production of cytokines and activating natural killer cells (23). These studies clearly indicated that bacterial DNA had immunomodulatory effects. Furthermore, these immunostimulatory properties of bacterial DNA were shown to be mainly due to a specific sequence of cytosine-phosphodiester-guanine (CpG) motifs. These motifs are present in the unmethylated state and occur at a higher frequency in bacterial DNA than in mammalian DNA (23). Further studies demonstrated that synthetic CpG-containing oligodeoxynucleotides (CpG-ODN) induce B-cell proliferation and immunoglobulin (Ig) secretion, monocyte secretion of the Th1-like cytokines interleukin-12 (IL-12) and α, β, γ interferon (IFN) secretion, and natural killer (NK) cell lytic activity (1), 3, (8, 12), (15), (19, 22, 23, 29). Furthermore, a number of studies in mammalian species indicate that CpG-ODN can shift the immune response to a Th1-dominated cytokine pathway, which explains the predominance of Th1-like responses when CpG-ODN is used as an adjuvant with subunit or conventional vaccines (4, 14, 31) but the two major arms of immunity, regulated by a T helper (Th1)/Th2 cytokine balance, evolved similarity in chickens is unknown.

It has been demonstrated that chicken macrophages (cell line HD11) and chicken peripheral blood mononuclear cells exposed to CpG-ODN have an increased ability to

kill intracellular *Salmonella enteritidis* (10) . Secretion of IL-6, IL1β, IFN-γ and nitric oxide production also were stimulated (9, 11, 27). When bovine serum albumin was used as a test antigen in combination with CpG-ODN, antibody production against bovine serum albumin was increased. Similarly, when CpG-ODN was combined with antigens of infectious bursal disease virus, antibody production was enhanced (24, 25).

Escherichia coli causes a variety of disease syndromes in poultry including yolk-sac infection, omphalitis, respiratory tract infection, septicemia, and cellulitis (17, 20). Cellulitis and other lesions caused by *E. coli* have been reproduced experimentally in chickens (7, 18). A percentage of experimentally infected birds develop colibacillosis, characterized in its acute form by septicemia resulting in death, and in its subacute form by pericarditis, airsacculitis, and perihepatitis (3). Although the pathogenesis of colibacillosis is poorly understood the development of bacteremia appears to be essential (3, 16).

Until now, the use of CpG-ODN as a vaccine adjuvant against bacterial diseases in poultry has not been reported. The objective of this study was to compare the active immune response of chickens to a killed *E. coli* vaccine combined with either oil-in-water emulsion or with CpG-ODN when challenged with homologus *E. coli*.

(c) METHODS

E. coli isolate and preparation of *E. coli* bacterin

A field isolate of *E. coli* from a turkey with colisepticemia was used. This *E. coli* was serogroup O2, nonhemolytic, serum-resistant and produced aerobactin, a K1 capsule and type 1 pili (6).

E. coli antigens were prepared from killed *E. coli*. Cells, derived from a frozen culture, were passaged once on TSA with 5% sheep blood and then grown aerobically at 37°C in brain heart infusion broth (BHI) for 12-14 h in a 50 ml Erlenmeyer flask, shaken at 200 rpm. A 1:10,000 dilution of the culture was made in 100 ml of BHI in a 1L Erlenmeyer flask. This culture was incubated for 8-9 h at 37°C, shaken at 200 rpm, until an absorbance of 0.8 at OD_{600} was reached. This produced a culture containing approximately $1x10^9$ cfu per ml in the logarithmic phase of growth. The bacterial cells were washed once with normal saline, resuspended in one volume of normal saline, and then incubated at 65°C for one hour. One ml of heat-killed *E. coli* culture suspension was pelleted by centrifugation and resuspended in 0.3 ml volumes of normal saline. Viability of *E. coli* after heat treatment was tested by plating 100 µl of antigen solusion on TSA with 5% sheep blood in duplicate.

Bacterin formulation and dose

Three vaccines were formulated. All of the vaccines contained 300 µl of the concentrated *E. coli* per dose. One vaccine contained 150 µl of VSA (oil in water emulsion [Emulsigen®] (Ralston, NE) with added dimethlydioctadecylammonium bromide per dose. A second vaccine contained 50 µg of CpG-ODN in normal saline. The third vaccine contained 10 µg of CpG-ODN in normal saline. Birds were inoculated, at 10 or 20 days of age, by subcutaneous injection into the neck.

Animal model of *E. coli*

All procedures involving animals were done with the approval of the University of Saskatchewan Committee on Animal Care. Day-old broiler chicks were obtained from a local hatchery in Saskatchewan, Canada and identified individually by wing bands.

Groups of chicks were randomly allocated into individual animal isolation rooms at the Vaccine and Infectious Disease Organization, University of Saskatchewan. Water and commercial broiler ration were provided *ad libitum*. Birds received 8 hours of light. Room temperature was maintained at 20-22° C. Filtered, non-recirculated air was provided at a rate of 10-12 changes/h. Air pressure differentials and strict sanitation conditions were maintained in this isolation facility.

The previously-described model for the production of cellulitis and colibacillosis in broiler chickens was used with few modifications (6.7). Briefly, at 30 days of age, two parallel 3 cm long full thickness scratches were made through the skin on the left caudal abdominal region with a diabetic lancet (Lifescan® Lancets, Lifescan Canada Ltd., Burnaby, B.C.). The *E. coli* challenge inoculum was prepared as described above and was applied to the scratches with 2 cotton applicators (Hardwood Products Company LLC, Guilford, Maine, USA) and was repeated 15 min later. In this model, cellulitis develops in 100% of birds. Pericarditis, airsacculitis, and perihepatitis develop in 50 – 90% of birds. Occasionally, a few birds also develop hepatitis. Mortality ranges between 50 – 90% in infected birds.

Birds were observed twice daily and scored for 10 days post-challenge for clinical signs of disease. Each individual was assigned a daily clinical score: 0 = normal; 0.5 = slightly abnormal appearance, slow to move; 1 = depressed, reluctant to move; 2 = unable to stand or reach food or water; 3 = found dead. Birds that received a clinical score of 2 were euthanized by cervical dislocation.

Gross pathological examinations and bacteriological cultures were done immediately on chickens that were found dead or euthanized. Bacterial swabs were taken

from the cellulitis lesion, pericardium, and air sacs and cultured on MacConkey (Difco) and trypticase soy agar (TSA) with 5% sheep blood (PML Microbiologicals, Richmond, B.C.) A semi-quantitative estimate of *E. coli* growth was conducted on MacConkey agar (5)(2). Any gross lesions were recorded. On day 10, the remaining birds were euthanized by cervical dislocation, and necropsy and bacteriological procedures were done as above.

Synthetic CpG-ODN.

The sequence of CpG-ODN[2007] used was TCGTCGTTGTCGTTTTGTCGTT. This CpG-ODN was free of endotoxin and contained a phosphorothioate backbone (Qiagen-GmbH, Hilden Germany). Synthetic CpG-ODN was diluted in sterile, pyrogen-free saline.

Development of ELISA to detect chicken IgG against whole cell *E. coli*

Immulon ®2, polystyrine 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C using 0.2 µg of killed whole cell *E. coli* antigen in 100 µl of coating buffer (0.05 M sodium carbonate, 0.05 M sodium bicarbonate, pH. 9.6) (BDH Inc. Toronto, ON) per well. The plates were washed six times with distilled water and blocked for 1.5 h with skim milk [Co-op skim-milk powder 1g in 20 ml of phosphate-buffered saline (pH 7.3), supplemented with 0.05% Tween-20 (PBST)]. Serial dilutions of test sera were added and incubated for 2 h at room temperature. After washing six times, as previously described, 100 µl of 1:5000 goat anti-chicken IgG (heavy and light chain) (Kirkegaard and Perry Laboratories Inc., Gaithersbury, MD) were added to each well. The plates were incubated at room temperature for 1.5 h, washed six

times and 100 µl of (p-Nitrophenyl Phosphate) (PNPP) (1 mg PNPP per ml of 1% Diethanolamine with 0.5 mM $MgCl_2$, Ph 9.8) (Sigma Chemical Co., St. Louis, MO) substrate was added. After incubation at room temperature for 1.5 h, the absorbance was measured at an optical density (OD) of 405/495 nm using a BioRad® Microplate reader (BioRad, Richmond, CA). The OD values were recorded as the mean of triplicate wells in each plate. Positive and negative control sera and blank wells with no sera, were used as controls.

Development of ELISA to detect chicken IgG against *E. coli* lipopolysaccharide (LPS)

Immulon® 2, polystyrine 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C using 10 EU of *E. coli* LPS (Sigma Chemical Co. St. Louis, MO) in 100 µl of coating buffer (0.05 M sodium carbonate, 0.05 M sodium bicarbonate, pH. 9.6) (BDH Inc. Toronto, ON) per well. The plates were washed six times with distilled water and blocked for 2 h at 37°C using 1% gelatin in phosphate buffered saline with 0.05% Tween-20 (PBST). Serial dilutions of the test sera were added and incubated for 2 h at room temperature. After washing six times, as previously described, 100 µl of 1:5000 goat anti-chicken IgG (heavy and light chain) (Kirkegaard and Perry Laboratories Inc., Gaithersbury, MD) were added to each well. The plates were incubated at room temperature for 1.5 h, washed six times, and 100 µl of p-Nitrophenyl Phosphate (PNPP) (1 mg PNPP per ml of 1% Diethanolamine with 0.5 mM $MgCl_2$, pH 9.8) (Sigma Chemical Co. St. Louis, MO) substrate was added. After incubation at room temperature for 1.5 h, the absorbance was measured at an optical density (OD) of 405/495 nm using a BioRad® Microplate reader (BioRad, Richmond, CA). The OD

values were recorded as the mean of triplicate wells in each plate. Positive (birds recovered from *E. coli* septicemia) and negative control (healthy birds) sera, and blank wells with no sera, were used as controls.

Experimental design

a) CpG-ODN as an adjuvant with *E. coli* bacterin vaccine.

All birds were housed together in one room, randomly allocated to groups of 25, identified with wing bands, and vaccinated either once at 10 days of age or once at 10 days of age and again at 20 days of age with one of the vaccines described above. One control group received saline injections at both 10 and 20 days of age. A second control subgroup received 50µg of CpG-ODN at 10 days of age only. The third control subgroup received 50 µg of CpG-ODN at both 10 and 20 days of age (Table 1). All the birds were challenged with homologous *E. coli* at 30 days of age as described above. The birds were examined for clinical signs of disease for 10 days after challenge. The remaining live birds were euthanized and examined 10 days after challenge. Serum samples were collected from ten randomly-selected birds at 20 and 30 days to assay total IgG against *E. coli* whole cell and LPS.

Table 1: Vaccination schedule of *E. coli* antigen together with CpG-ODN or VSA as an adjuvant.

Groups	**Subgroups**	**primary vaccination**	**secondary vaccination**
1. Control	Saline	Yes	Yes
	50 µg CpG-ODN	Yes	No
	50 µg CpG-ODN	Yes	Yes
2. VSA as an adjuvant	Bacterin + VSA	Yes	No
	Bacterin + VSA	Yes	*Yes*
3. CpG-ODN as an adjuvant	Bacterin + 10 µg CpG-ODN	Yes	No
	Bacterin + 10 µg CpG-ODN	Yes	Yes
	Bacterin + 50 µg CpG-ODN	Yes	No
	Bacterin + 50 µg CpG-ODN	Yes	Yes

b) Evaluation of tissue reactions at the site of CpG-ODN administration.

Individual groups of fifteen birds received saline, 50 μg CpG-ODN, or 30% VSA by intramuscular injection into the left drumstick at 14 days of age. Three birds from each group were euthanized on 1, 3, 7, 10 and 14 days post injection. Tissue samples from the injection site were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness, and stained with hematoxylin and eosin (H&E). A histological score from 0 to 4+ (0 = no visible lesions; 1+ = focal, mild infiltrations of mononuclear leukocytes and heterophils or mild myositis; 2+ = locally extensive, moderate infiltrations of mononuclear leukocytes and heterophils, or moderate myositis; 3+ = locally extensive, moderate to severe infiltrations of mononuclear leukocytes and heterophils,or necrotizing moderate to severe myositis; 4+ = diffuse, severe infiltrations of mononuclear leukocytes and heterophils, or severe necrotizing, hemorrhagic myositis) was assigned.

Statistical analysis.

The survival pattern and median survival were compared using the logrank test and Chi-square statistics. Survival data were analyzed using Prism (Prism 3.0,GraphPad Software Inc., San Diego, CA. USA www.graphpad.com) with a significance level of p = 0.05. The significance of effect of treatment group on *E. coli* isolation score was determined by contingency table analysis (crosstabulation program) and Chi-Squire test or Fisher's exact test as appropriate using SYSTAT 10.2, (SYSTAT Software Inc. 501 Canal Blvd, Suite C Point Richmond, CA 94804-2028 USA. info-usa@systat.com.

(d) RESULTS

a) CpG-ODN as an adjuvant with *E. coli* bacterin vaccine

Birds vaccinated once or twice with *E. coli* bacterin using CpG-ODN as an adjuvant were significantly better protected against homologous *E. coli* challenge ($p<0.05$) compared to control groups. In contrast, birds vaccinated with *E. coli* bacterin and VSA were not protected against *E. coli* challenge ($p>0.05$) (Fig. 1). Furthermore, birds vaccinated with *E. coli* bacterin adjuvanted with CpG-ODN were significantly better protected against *E. coli* infection compared to the birds vaccinated with *E. coli* bacterin adjuvanted with VSA ($P<0.05$). For all nine subgroups (Table 1) of the variables bacterin, CpG-ODN, CpG-ODN dose, VSA, and number of vaccinations, only the variable bacterin had a significant effect on survival following homologous *E. coli* challenge ($p=0.001$); hence the protection that was demonstrated is the result of active immunization. Of the six subgroups (Table 1) that were given bacterin, only the group with CpG-ODN had a significant positive effect increasing survival after *E. coli* challenge ($p<0.05$; relative risk was 0.41). Among the subgroups containing CpG-ODN, there was no significant effect against *E. coli* challenge either due to number of administrations of CpG-ODN ($p=0.9$) or the dosage of CpG-ODN ($p = 0.7$). The cumulative total clinical scores were 729 for the controls, 537 for the bacterin with VSA, and 482 for the bacterin with CpG which was the lowest.

IgG antibody (Ab) titer against *E. coli* whole cell bacterin was less than $1x10^2$ at 20 days of age after one vaccination with no difference in Ab levels among the groups at that time (data not shown). However, antibody titers increased at 30 days of age, in groups that had received a second vaccination of bacterin containing either VSA or CpG-

ODN (Fig. 2). Groups vaccinated with the *E. coli* bacterin and either CpG-ODN or VSA had significantly higher titers compared to the control groups ($p<0.05$). There was no statistical difference in titers between groups treated with bacterin containing VSA or CpG-ODN ($p>0.05$) regardless of the dose of CpG-ODN or if given 1 dose versus 2 doses (Fig. 2). IgG levels against LPS were less than $1x10^2$ in all groups vaccinated either once or twice, and there was no statistical difference among groups ($p>0.05$) (data not shown).

Escherichia coli was cultured from lesions of cellulitis in 28% of birds that had been vaccinated with the bacterin containing CpG-ODN. This was a significantly lower isolation rate ($p < 0.01$) than in the group treated with VSA (57%) or in the controls (51%). Likewise, the proportion of birds with *E. coli* in air sacs was significantly lower ($p < 0.05$) in the groups treated with CpG-ODN (6%) than in the group treated with VSA (19%) or in the controls (23%). There was no difference in the level of isolation of *E. coli* from the pericardium among the groups ($p>0.1$) (Table 2).

b) Evaluation of tissue reactions at the site of vaccine adjuvant administration.

Since tissue damage induced by vaccine adjuvants is an important consideration, a study was conducted to examine the tissue response at the site of injection (Fig. 3). In birds that received CpG-ODN by intramuscular injection, cellular infiltration was moderate during the first week and had disappeared by 14 days post-inoculation. In contrast, severe cellular infiltration, hemorrhage, edema, and necrosis were noticed during the first four days following inoculation in birds that received 30% VSA, and

myofiber regeneration was noticed within 14 days post-inoculation (Fig. 3). No tissue reaction was observed at the injection site in birds receiving only saline.

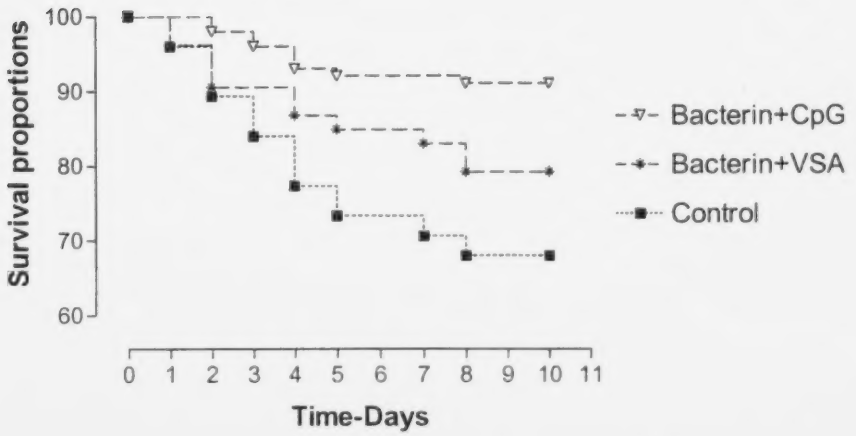

Fig: 1: Survival of broilers after *E. coli* challenge. Birds vaccinated with *E. coli* bacterin adjuvanted with CpG-ODN once or twice were significantly protected against *E. coli* challenge. ($p<0.05$). In contrast, birds vaccinated with *E. coli* bacterin adjuvanted with VSA were not protected against *E. coli* challenge ($p>0.05$) at a significant level. Furthermore, birds vaccinated with the *E. coli* bacterin adjuvanted with CpG-ODN were significantly protected against *E. coli* infection compared to the birds vaccinated with *E. coli* bacterin adjuvanted with VSA ($P<0.05$). [Bacterin = *E. coli* bacterin; VSA = oil in water emulsion (Emulsigen + DDA); Control = saline or CpG-ODN alone]. Results are shown by combination of groups as described in Table 1.

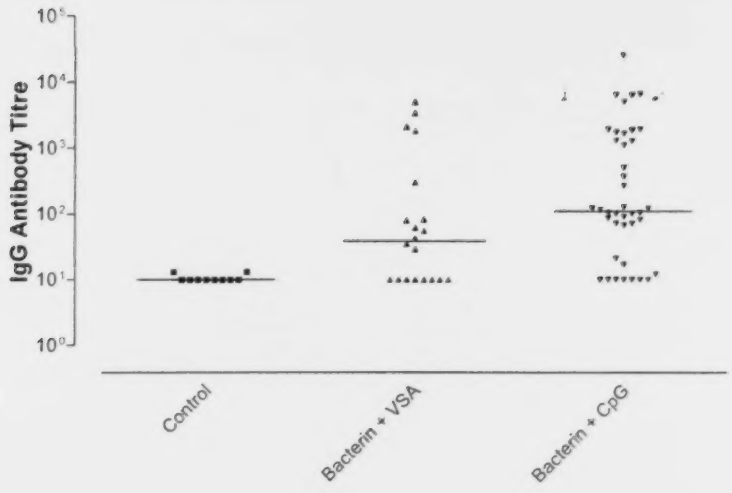

Fig. 2: Total IgG against *E. coli* bacterin in individual birds and median at day 30 after secondary vaccination. [Control = saline or CpG-ODN alone; Bacterin + VSA = *E. coli* bacterin adjuvanted with oil in water Emulsigen® (Emulsigen + DDA); Bacterin + CpG = *E. coli* bacterin adjuvanted with CpG-ODN]

Table 2: Bacterial isolations from air sacs, pericardium and cellulitis lesion of *E. coli* infected birds.

Bacterial isolation site	[a]*E. coli* growth score	% of birds in the treatment group		
		Control (%)	Bacterin + VSA (%)	Bacterin + CpG-ODN (%)
Cellulitis	0	[b] 49	43	72
	1+	28	42	22
	2+	10	9	6
	3+	9	2	0
	4+	3	4	0
Air sacs	0	77	81	94
	1+	12	17	6
	2+	7	2	0
	3+	0	0	0
	4+	3	0	0
Pericardium	0	81	89	92
	1+	4	6	6
	2+	5	4	2

The proportion of birds with no *E. coli* in cellulitis lesions was significantly ($p < 0.01$) greater in the group treated with CpG-ODN (72%) than in the group treated with VSA (43%) or the controls (49%). Likewise, the proportion of birds with no *E. coli* in air sacs was significantly ($p < 0.05$) greater in the group treated with CpG-ODN (94%) than in the group treated with VSA (81%) or the controls (77%).

[a] Estimate of *E. coli* concentration in lesions was measured on MacConkey agar by streaking sequentially in 4 distinct areas on agar plates. The enumeration was expressed on a 0 to 4+ scale (0 = no growth; 1+ = growth of bacteria on the area 1; 2+ = growth of the bacteria on area 1 and 2; 3+ = growth of bacteria on area 1, 2 and 3; 4+ = growth of bacteria on area 1, 2, 3 and 4). [b] % of birds in group with corresponding bacterial score.

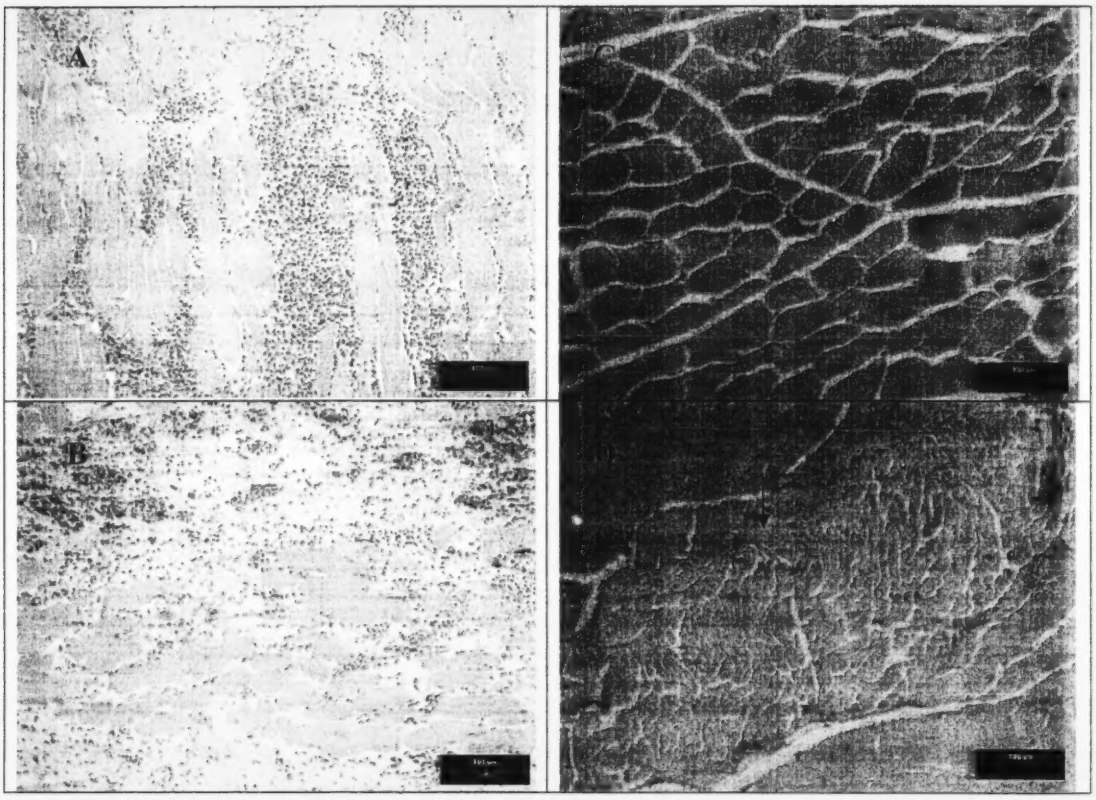

Fig. 3: Tissue reactions following 24h and 14 days of injecting CpG-ODN or oil in water emulsion with DDA [VSA]. (A) Infiltration of both heterophils and mononuclear cells at a moderate degree at the site of 50 µg of CpG-ODN injection at 24 h. (B) Severe hemorrhage, edema, necrosis and infiltration of inflammatory cells at the site of injection of 30% VSA [oil in water emulsion with DDA (Dimethlydioctadecylammonium Bromide)] at 24 h. (C) Normal tissue at 14 days following CpG-ODN injection. (D) Newly formed muscle fibers (myofiber regeneration) (arrow) at 14 days following 30% VSA injection. Bar = 100 µm.

(e) CONCLUSIONS AND RECOMENDATIONS

Synthetic oligodeoxynucleotides containing CpG motifs act as immune adjuvants, accelerating and boosting antigen-specific antibody responses. Bacterial DNA with unmethylated CpG-ODN induces maturation and activation of vertebrate immune cells which then produce TNF-α, Th1-type cytokines, IL-12, and IFN-γ. Therefore, CpG-ODN functions as an adjuvant to initiate Th1 lymphocyte differentiation (13, 28). The inclusion of CpG-ODN in a variety of vaccines has increased protection against challenge in animal models (26). Ongoing clinical studies indicate that CpG-ODNs are safe and well-tolerated when administered as adjuvants to humans and, in some cases, increase vaccine-induced immune responses (5, 13).

The effect of CpG-ODN as an immunostimulant against septicemia in chickens has been demonstrated previously (6, 10), but the effect of CpG-ODN as a vaccine adjuvant against bacterial diseases in poultry has not been described. This study demonstrates that CpG-ODN is an effective vaccine adjuvant with an *E. coli* bacterin and significantly protected birds against a challenge of *E. coli* capable of causing septicemia and cellulitis in unvaccinated birds. The *E. coli* bacterin containing an oil-in-water adjuvant was not able to protect birds against homologous *E. coli* challenge at a significant level. Clinical signs and bacterial counts of skin (cellulitis) and air sac lesions of birds correlated with their survival (Table 2). A significant difference was not observed when the concentration of CpG-ODN was increased from 10μg to 50μg. CpG-ODN, at the10μg concentration may provide the maximum response possible. It will be necessary to test lower doses of CpG-ODN to confirm this observation.

Total IgG against an *E. coli* bacterin was increased after a second vaccination. This phenomenon could be partly correlated to protection. Although cell-mediated immunity (CMI) was not studied in these experiments, it is likely CMI is partly responsible for protection of birds against *E. coli* infection. This hypothesis is further supported by the observation that birds receiving *E. coli* bacterin containing CpG-ODN adjuvant showed greater protection against a challenge of *E. coli*, than birds receiving *E. coli* bacterin containing an oil-in-water adjuvant although total IgG levels were not statistically different between these groups. Although, *E. coli* LPS has been demonstrated to be a protective antigen in other studies (21), we did not see any significant antibody response to LPS in birds that received the *E. coli* bacterin. It is possible that the dose of *E. coli* LPS contained in the bacterin was not optimal in this study.

It has been previously reported by our group that CpG-ODN effectively stimulates innate immunity against cellulitis and colibacillosis in chickens. This activation of innate immunity lasts only for about six days (6). Here, it has been demonstrated that 2) days after vaccination with the same Class B CpG-ODN, protection still remained against *E. coli* challenge. These studies further demonstrated that CpG-ODN could elicit innate and adaptive immune responses in poultry.

It has been previously reported that CpG-ODN elicited protection as an immunostimulant and does not cause tissue necrosis (6) at the site of injection . In mice, it has been suggested that CpG-ODN failed to activate mast cell degranulation (30) and hence caused no tissue necrosis. Here, we have demonstrated that CpG-ODN is an effective adjuvant when used with a conventional vaccine and does not cause tissue necrosis. In contrast, vaccines containing oil-in-water emulsion (VSA) are less effective

and cause tissue necrosis (Fig. 3). Based on these observations, we believe CpG-ODN to be a safe and effective adjuvant for use in the poultry industry.

It is generally accepted that it is better to prevent disease than to treat it. This has become especially important due to a number of food safety and human health issues. The use of antibiotics by the animal industry has been linked to the emergence of resistant strains of bacteria. Also, the issue of drug residue in meat products is important, both for domestic and international trade. For these reasons, the animal production industry is seeking to reduce the use of therapeutic agents by developing effective immunomodulators. CpG-ODN has already been tested in human vaccines (5). Based on these studies we conclude that CpG-ODN has great potential for use as an adjuvant in modern poultry vaccines.

SUMMARY – (work completed in year 1 & 2) A manuscript has been submitted to *Current Drug Delivery*

Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to be effective immunoprotective agents and vaccine adjuvants in a variety of bacterial and protozoan diseases in different animal species. The objective of this study was to investigate the immunoprotective effect of formulated CpG-ODN with polyphosphazene, liposome or oil-in-water emulsion against *E. coli* infections in neonatal chickens. Eighteen-day-old embryonating eggs were inoculated with 50 μg CpG-ODN or formulated CpG-ODN with polyphophazene, liposome or oil-in-water emulsion. Four days after exposure to formulated CpG-ODN or day-1 post-hatch, $1x10^4$ or $1x10^5$ cfu's of a virulent isolate of *E. coli* was inoculated by the subcutaneous route in the neck. Clinical

signs, pathology, bacterial isolations from the air sacs, and mortality were observed for eight days following challenge with *E. coli*. The survival rate of birds following *E. coli* infection was 0% in groups receiving either non-CpG-ODN or saline. In contrast, birds receiving either CpG-ODN or CpG-ODN formulated with polyphosphazene had significantly higher survival of 55% ($P<0.0001$). The relative risk of mortality was significantly reduced for birds treated with CpG-ODN formulated in PCPP (0.25), in PCEP (0.33), or unformulated CpG-ODN (0.39) in comparison to the group treated with saline ($p<0.01$). Although formulation of CpG-ODN with liposomes or oil-in-water emulsion did not increase the immunoprotective effect against *E. coli* infection, no adverse reactions or poor hatchability were observed in embryos. This is the first time that CpG-ODN formulated with polyphosphazene has been demonstrated to have an immunoprotective effect against an extra cellular bacterial infection in neonatal broiler chickens following *in ovo* delivery.

SUMMARY – "Effect of CpG-ODN against *S. typhimurium* infection in neonatal broiler chicken and *in ovo* delivery" – A manuscript has been submitted to *Infection and Immunity*

Oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to stimulate the innate immune system against a variety of bacterial and protozoan infections in a variety of vertebrate species. The objective of this study was to investigate the immunostimulatory effect of CpG-ODN in neonatal broilers against *Salmonella typhimurium* septicemia. Day old broiler chicks, or embryonated eggs that had been incubated for 18 days, received 50 μg of CpG-ODN, 50 μg of non-CpG-ODN, or saline.

Four days after exposure to CpG-ODN or day 2-post hatch, $1x10^6$ or $1x10^7$ cfu of a virulent isolate of *S. typhimurium* was inoculated by the subcutaneous route in the neck. Clinical signs, pathology, bacterial isolations from the air sacs, and mortality were observed for ten days following challenge with *S. typhimurium*. The survival rate of birds in groups receiving either non-CpG-ODN or saline following *S. typhimurium* infection was 40-45%. In contrast, birds receiving CpG-ODN had significantly higher survival rate of 80-85% ($P<0.0001$). Bacterial loads and pathology were low in groups treated with CpG-ODN compared to the groups receiving saline or non-CpG-ODN. Colony forming units of *S. typhimurium* in the peripheral blood were significantly lower in birds treated with CpG-ODN compared to the group that received saline. This is the first time that CpG-ODN has been demonstrated to have an immunoprotective effect against an intracellular bacterial infection in neonatal broiler chickens following *in ovo* delivery.

SUMMARY – Mechanism of action of CpG-ODN in neonatal chickens – A manuscript has been submitted to *Developmental & Comparative Immunology*.

Earlier, we demonstrated that intramuscular administration of oligodeoxynucleotides containing CpG motifs (CpG-ODN) induces protection in neonatal chicks against a lethal challenge of *Escherichia coli*. However, the mechanism of induction of the protection was not clear. In an attempt to elucidate the mechanism of induced protection, we determined the kinetics of expression of cytokines/chemokines in the spleen and bursa of Fabricius of newly hatched chicks that had received intramuscular administration of CpG-ODN or non CpG-ODN compared to saline-treated controls. SyBr green, real-time, quantitative reverse transcription polymerase chain reaction (RT-

PCR) analysis of the RNA demonstrated increased expression of IL-1β, IL-6, IL-8, IL-10, IL-18, IFN-γ and MIP-3α mRNAs in the spleen of chicks that had received CpG-ODN. However, there was no significant difference in the mRNA levels of any cytokine in the bursa of Fabricius of treated chicks, except IFN-α, which was downregulated in chicks that had received either CpG-ODN or non-CpG ODN. The increased level of IL-18 and IFN-γ but not IL-4 mRNA suggests that the administration of CpG-ODN elicits a Th1 biased immune response, which may be important in inducing protection against infections in neonatal chicks. To our knowledge, this is the first report evaluating the induction of cytokines/chemokines in neonatal chicks following administration of CpG-ODN.

(f) Acknowledgements

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(i) ADMINISTRATIVE AND OTHER ASPECTS

Training of highly qualified personnel:

Azita Taghavi, a graduate student will be defending her M.Sc. thesis on Oct. 23, 2007.

Bhavini Parekh, a graduate student in a PhD program conducted studies to understand the mechanism of action CpG-ODN in chickens.

Anjela Oranchuk (currently a 2nd year undergraduate student in the DVM program) assisted laboratory work and animal experiments during summer months of 2005 and 2006 as a research technician.

INVITED PRESENTATIONS:

Susantha Gomis, 78th Northeastern Conference on Avian Diseases, Ontario Veterinary College, University of Guelph. "Innate immunity, its utility and links with adaptive immunity" June 15 – 16, 2006

Susantha Gomis, Poultry Industry Council, Guelph, Ontario. "Research Day 2005". "Immunostimulatory effect of CpG-DNA" Guelph, ON. May 18, 2005.

MANUSCRIPTS:

Azita Taghavi, Brenda Allan, Philip Willson, and **Susantha Gomis**. Experimental reproduction of *Salmonella typhimurium* infection in neonatal broiler chickens. (A manuscript in preparation – *Avian Dis*)

Taghavi, A., Allan, B., Mutviri, G., Van Kessel, A., Babiuk, L., Potter, A. Willson, P., and **S. Gomis**. Delivery of CpG-ODN in incubating eggs protects neonatal chickens against a lethal challenge of *Salmonella typhimurium* septicemia. (Manuscript submitted to – Infection and Immunity)

Taghavi, A., Allan, B., Mutviri, G., Van Kessel, A., Marianna Foldvari, M. Babiuk, L., Potter, A. Willson, P., and **S. Gomis**. Oligodeoxynucleotides containing CpG motifs (CpG-ODN) formulated with polyphophazines and lipid based delivery systems enhanced the immunoprotective effect against *Escherichia coli* septicemia in neonatal chickens following *in ovo* delivery. (Manuscript submitted to - Curr Drug Deliv)

Parekh, B., **Gomis, S.**, Dar, A., Willson, P., Babiuk, L., Potter, A., G. Mutwiri, and S. Tikoo. Oligodeoxynucleotides containing CpG motifs (CpG-ODN) induced cytokine and chemokine gene expression in neonatal chickens. (Manuscript submitted to - Developmental and comparative immunology)

Gomis, S., Babiuk, L., Allan, B., Willson, P., Waters, E., Hecker, R., and A. Potter. Protection of chickens against a lethal challenge of *Escherichia coli* by a vaccine containing CpG oligodeoxynucleotides as an adjuvant. Avian Dis. 51:78-83. 2007.

PRESNTATIONS IN NATIONAL AND INTERNATIONAL CONFERENCES:

A. Taghavi, B. Allan, G. Mutwiri, A. P. Willson, Van Kessel, L. Babiuk, A. Potter, and **Gomis, S**. Protection against a lethal challenge of *S. typhimurium* septicemia in neonatal chickens with CpG-ODN. 18th Western Meeting of Poultry Clinicians and Pathologists "WestVet 18" Oct. 1-2, 2007. Lake Louise AB.

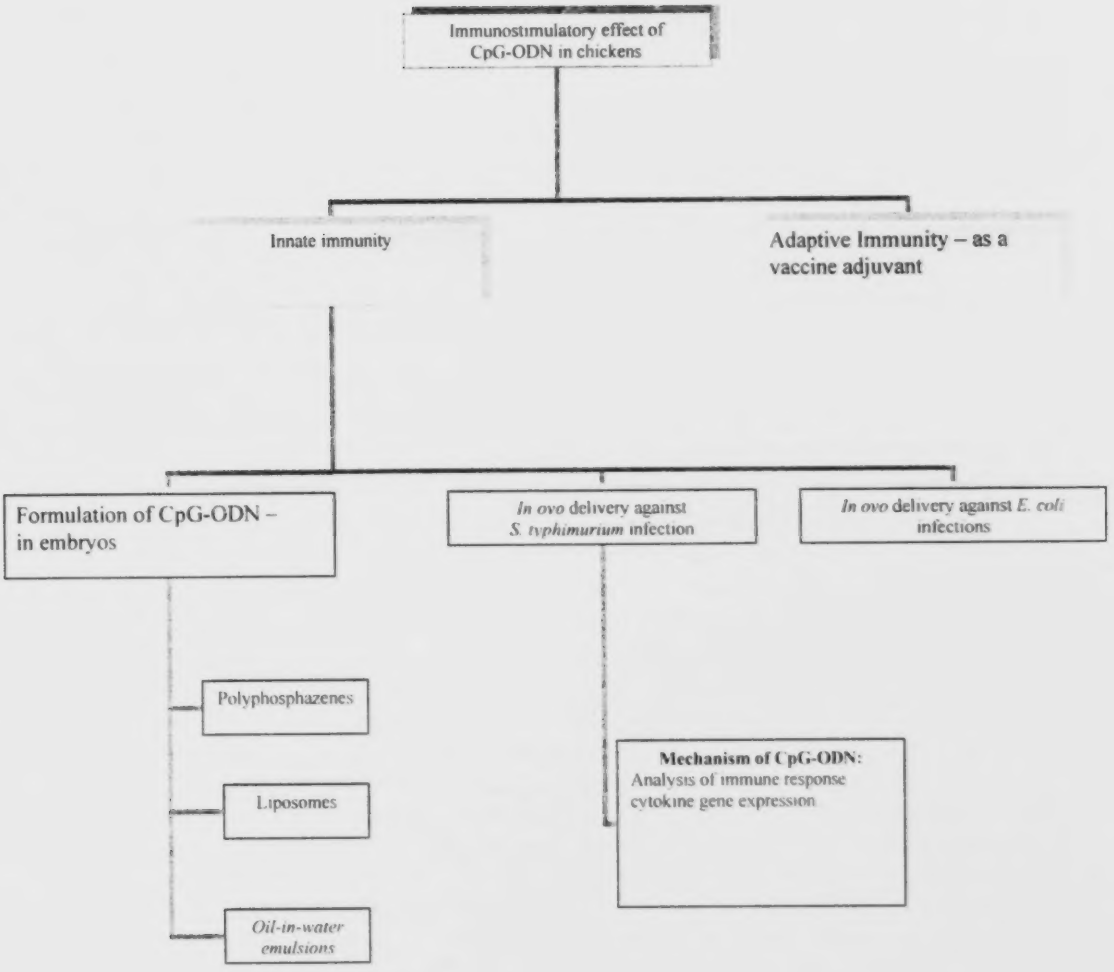
B. Parekh, **S. Gomis** , A. Dar, P. Willson, L. Babiuk, A. Potter, G. Mutwiri, and S. Tikoo. Oligodeoxynucleotides Containing CpG Motifs (CpG-ODN) Induce Predominantly a Th1 Type Immune Response in Neonatal Chickens. American Association of Avian Pathologists/American Veterinary Medical Association, July 14-18, 2007. Washington DC.

Taghavi, A., B. Allan, G. Mutwiri, A. Van Kessel, L. Babiuk, A. Potter, and **S. Gomis** Oligodeoxynucleotides containing CpG motifs (CpG-ODN) confer protection against a lethal challenge of *Salmonella typhimurium* septicemia in neonatal broilers. American Association of Avian Pathologists/American Veterinary Medical Association, July 14-18, 2007. Washington DC.

Gomis S., Effect of synthetic bacterial DNA as an immunostimulant in broilers. Chicken Industry Invesment Fund and Development Fund, Annual General Meeting. Feb. 28, 2007. Saskatoon. SK.

A. Taghavi, B. Allan, G. Mutwiri, A. Van Kessel, L. Babiuk, A. Potter, and **S. Gomis.** Oligodeoxynucleotides containing CpG motifs (CpG-ODN) confer protection against a lethal challenge of *Salmonella typhimurium* septicemia in neonatal broilers. 78th Northeastern Conference on Avian Diseases, Ontario Veterinary College, University of Guelph. June 15 – 16, 2006

Gomis S., Babiuk L., Waters E., Willson P., Allan B., Hecker R., and A. Potter. Oligodeoxynucleotides containg CpG motifs (CpG-ODN) as a vaccine adjuvant in poultry. American Association of Avian Pathologists/American Veterinary Medical Association, July 16-20, 2005, Minneapolis, MN.


Immunostimulatory effect of CpG-ODN in chickens
Innate immunity
Adaptive Immunity – as a vaccine adjuvant
Formulation of CpG-ODN – in embryos
In ovo delivery against S. typhimurium infection
In ovo delivery against E. coli infections
Polyphosphazenes
Liposomes
Oil-in-water emulsions
Mechanism of CpG-ODN: Analysis of immune response cytokine gene expression